**EXHIBIT RAS-9**

This is exhibit RAS-9 referred to in Declaration Under 37 C.F.R. 1.132 by Richard Anthony Strugnell dated 27.9.01

Richard Strugnell

density determinant) and *positive* when coating was with Sp3 (recognizing a *low* density determinant).[6] The precise reasons for the effect may involve structural features, but also the precise ratios of molecular species become critical because the reagents are monoclonal. Complex mixtures are to a certain extent self-correcting, because different antibody species present at different concentrations can act independently of each other. The use of preparations containing mixed molecules secreted by HLGK or HLK clones (Fig. 2) introduces further complications.

Precipitation analysis of labeled monoclonal antibodies mixed with polyvalent antisera is a method that is likely to be used extensively. It was observed that under these conditions the labeled monoclonal antibodies were able to diffuse through the precipitin lines to which they bind and coprecipitate with another line. This is contrary to the old assumption that precipitin lines act as diffusion barriers. One monoclonal antibody in excess may not be able to dissolve the precipitate and diffuse through it to bind to other precipitin lines containing the same determinant on a different molecular species.[22]

The fine specificity of monoclonal antibodies is a great asset but should be used with caution. Negative results with a monoclonal antibody do not prove absence of the antigen itself. Changes in the environment of the antigenic determinant, or of the way the antigen is presented, could alter results. On the other hand, reaction with a monoclonal antibody could, at least in theory, occur through recognition of more than a single antigenic structure. More commonly the same antigenic determinants could be expressed in different molecular species—e.g., carbohydrate moieties or structural features in evolutionarily related proteins.



## [2] Production of Antisera with Small Doses of Immunogen: Multiple Intradermal Injections

*By* JUDITH L. VAITUKAITIS

A wide variety of immunization techniques has been used to generate specific antisera in laboratory animals. Those techniques incorporate a variety of injection routes, vehicles, and frequencies of injection into appropriate laboratory animals. Moreover, the concentrations of immunogen have ranged from gram to milligram concentrations. With the advent of more sophisticated isolation techniques, as well as the capacity readily to synthesize polypeptides, successful immunization with small amounts of immunogen has become imperative. Consequently, an approach to



generate specific antibody with high titer and affinity, using several micrograms of immunogen, became an obvious advantage.[1,2] Furthermore, the use of small quantities of immunogen is more likely to induce populations of antibody with high affinity and, consequently, sensitivity for the substance injected.[3]

*Principle.* Administer a water-in-oil emulsion containing the immunogen intradermally over a wide anatomic area in order to recruit many lymph nodes in the processing of immunogen for subsequent stimulation of antibody generation.

### *Reagents*

Buffer
Immunogen: 20–100 μg of highly purified polypeptide, polysaccharide, polynucleotide, or hapten conjugate
Freund's adjuvant
Dried, heat-killed tubercle bacillus

### Immunization

The water-in-oil emulsion to be injected is prepared as follows. The immunogen is initially dissolved in a buffer at an appropriate pH and molarity to enhance solubilization of the immunogen in that aqueous solution. An equal volume of that solution is combined with Freund's complete or incomplete adjuvant. When using Freund's complete adjuvant, which contains per milliliter 2 mg of heat-killed tubercle bacillus, one must be certain that the insoluble *Mycobacterium* is suspended in the oil. The *Mycobacterium* is usually precipitated to a small spot on the side or bottom of the vial. It may be suspended with gentle tapping. Additional heat-killed tubercle bacillus are added so that 2 ml of the subsequent emulsion will contain a total of 5 mg of heat-killed tubercle bacillus. Freund's incomplete adjuvant does not contain *Mycobacterium;* consequently, 5 mg of heat-killed tubercle bacillus need be added per 2 ml of the emulsion. It is important that an adequate water-in-oil emulsion be attained. The aqueous and oil phases of the preparation should not separate on standing. We have used a Sorvall Omnimixer at its maximum setting for 5–15 min or until the reaction mixture thickens and forms peaks when stroked with a glass rod or spatula. If prolonged mixing is required, the cannister containing the reagents should be bathed in iced water to pre-

[1] J. L. Vaitukaitis, J. B. Robbins, E. Nieschlag, and G. T. Ross, *J. Clin. Endocrinol. Metab.* 33, 988 (1971).
[2] J. L. Vaitukaitis and G. T. Ross, *Isr. J. Med. Sci.* 10, 1280 (1974).
[3] H. N. Eisen and G. W. Siskind, *Biochemistry* 3, 996 (1974).

vent heat-induced denaturation of the immunogen. The syringe-transfer technique frequently does not result in good emulsification of the reagents; consequently, a motor-driven blender is preferred. The emulsion, which resembles meringue or mayonnaise, is transferred to a syringe that has had the plunger removed. After loading the syringe from the back, the plunger is reinserted. The calibrated syringe facilitates the intradermal administration of 2 ml of the emulsion at multiple sites, usually with a 21-gauge needle, to each animal. We have used between 2 and 100 $\mu$g of immunogen with this technique. The immunogenicity of antigens varies considerably. Consequently, antibody may be generated with a single immunizing dose of 10 $\mu$g of one protein but only 50 $\mu$g of another.

Selection of an appropriate laboratory animal for immunization is important. One of the primary factors governing selection of the animal species is the antigenic similarity between the substance to be injected and that present in the animal species to be injected. As a general rule, the more structural dissimilarity between the two antigens, the more likely the animal will generate antibody to the injected substance. For most human immunogens, nonpregnant female rabbits suffice. For poorly understood reasons, pregnant animals are much less likely to generate antibody than their nonpregnant counterparts. Rabbits are relatively inexpensive and easy to handle, and more than adequate volumes of serum may be harvested from them on a weekly basis. Consequently, that species is frequently selected for antibody generation. Obviously, other animals may be selected for a variety of reasons. The two most common sites to harvest antibody from rabbits are the central ear artery and heart. Larger animal species—horse, sheep, goat—are usually bled from the jugular vein.

Figure 1 schematically depicts a rabbit shaved of fur along the back and proximal limbs. If larger animals are used, areas approximating 100 square inches may be shaved at the junctures of the back and proximal limbs, sites enriched with lymph nodes. Approximately 30–50 $\mu$l of the emulsion is injected at each site over the shaved area. Each animal receives 40–70 intradermal injections. Some investigators have injected the emulsion into the toe pads of animals, with successful generation of antisera with small doses of immunogen; however, animals immunized with that technique are more likely to develop "cage paralysis" with its attendant complications, including decreased antiserum production. Although antibody generation may become evident as early as 3 weeks after the primary immunization, it is usually not worthwhile to screen the sera until 6–8 weeks after the primary immunization. If antibody is detectable, then one should continue to bleed the animal on a weekly basis and assess the antisera for titer (antibody concentration) and sensitivity (affinity). Generally speaking, peak affinity is initially attained between weeks 8 and 10 after the primary immunization. If adequate sensitivity and specificity are





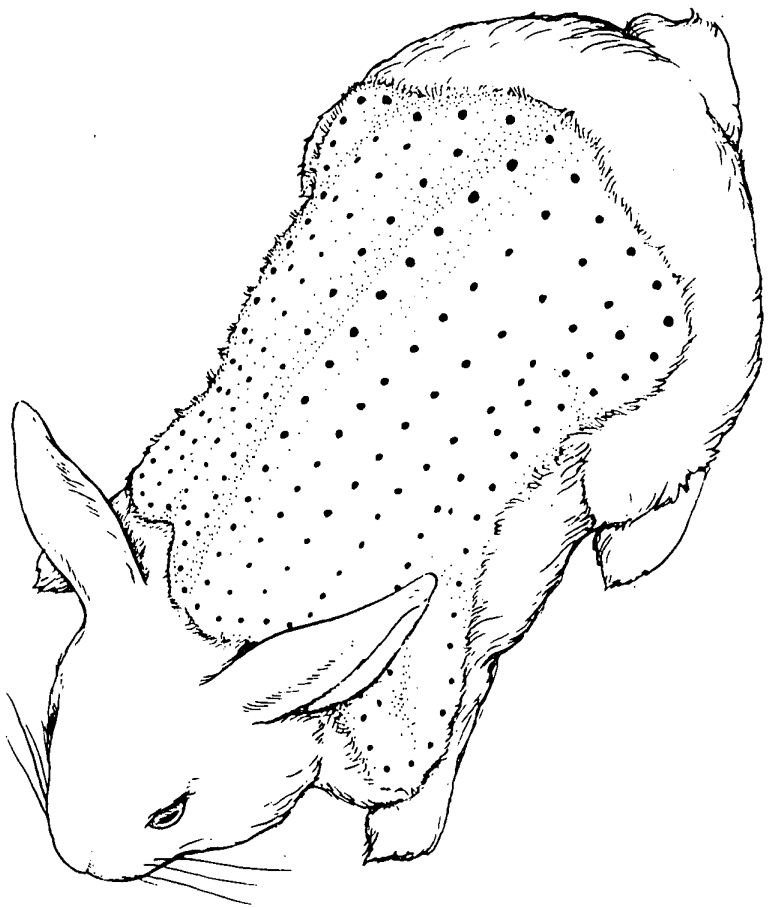

FIG. 1. Schematic drawing of a rabbit shaved of fur on its back and proximal limbs. The black dots over the shaved area represent intradermal injection sites of 30–50 $\mu$l.

not attained at that time, it is best to immunize another set of animals, since the sensitivity and specificity will not improve after that time. On the other hand, if no antibody is detectable at 6 weeks, the animal should be reimmunized with an emulsion devoid of tubercle bacillus and containing approximately one-quarter to one-half the amount of immunogen initially used. Those reimmunized animals can then be screened for antiserum production 10 days to 2 weeks after the reimmunization. Generally speaking, antibody titers remain at high levels with adequate specificity and sensitivity for several weeks to months after injection with a complete immunogen, in contrast to just several weeks with hapten conjugates.

Obviously, the technique used to characterize the antibody is important. For example, the Ouchterlony technique is approximately 100-fold

less sensitive than radioimmunoassay; moreover, precipitating antibody must be generated in order for precipitin lines to develop with the Ouchterlony technique. Significant cross-reactivity with contaminating or homologous proteins may be present, but the Ouchterlony technique may be too insensitive to detect them. The radioimmunoassay approach, on the other hand, is considerably more sensitive and specific, but more cumbersome and time consuming. Over the past several years, several new immunologic techniques with varying sensitivities have been introduced. The investigator must select the appropriate technique in terms of both sensitivity and specificity.

Figure 2 depicts serial titers over an 8-month period of four different animals immunized with 20 $\mu$g of hCG-$\alpha$, one of the two dissimilar subunits of hCG. A significant decline of circulating antibody was observed between 75 and 90 days after primary immunization with spontaneous restoration of higher titers after that time without reimmunization. The decline in titer was not attributable to interassay variation. It is interesting to note that one of those animals continued to generate high titers of antiserum for more than 8 months after the primary immunization.

In general, animals should not be "boosted" until a consistent decline of antibody titer is observed. Immunizations at too frequent intervals or with "emulsions" that separate on standing may result in tolerance. If the titer is decreasing, one may reimmunize the animal and may observe a significant rise in circulating antibody concentrations within 10–14 days after the "booster" injection. Moreover, the titers attained at this time usually markedly exceed the peak titers observed after the primary immunization. Figure 3 depicts circulating antibody titers in response to the

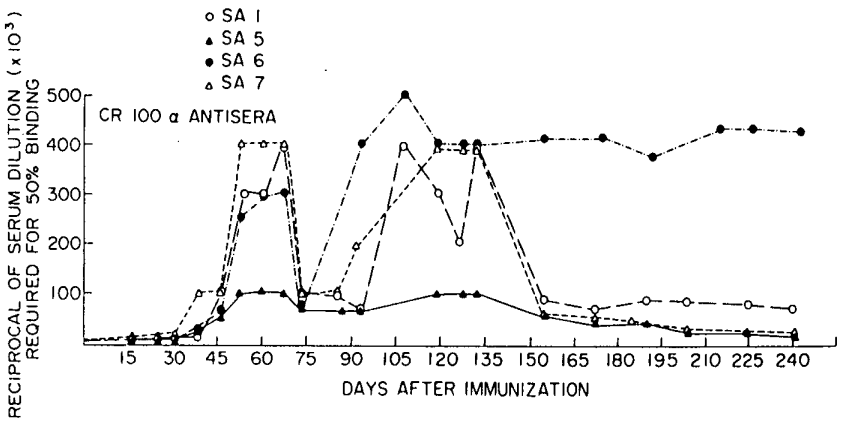


FIG. 2. Serial antibody titers of four animals immunized with 20 $\mu$g of highly purified hCG-$\alpha$. The titers were ascertained with [$^{125}$I]hCG-$\alpha$, corrected for nonspecific binding. All titers were determined on a single double antibody radioimmunoassay.



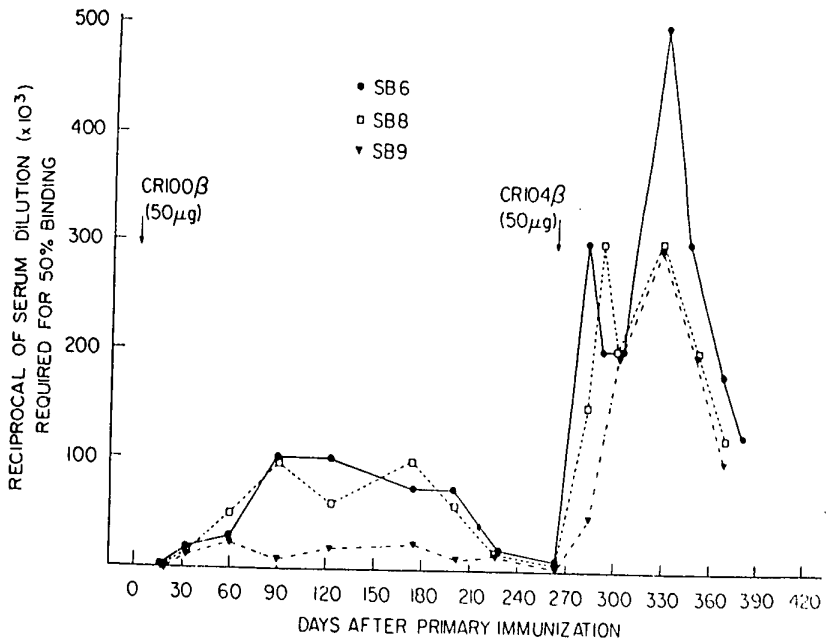


FIG. 3. Serial antibody titers among three animals immunized initially with 50 $\mu$g of highly purified hCG-$\beta$. The titers were determined by radioimmunoassay using a double antibody technique. Serum dilutions that bound 50% of counts per minute of [$^{125}$I]hCG-$\beta$, corrected for nonspecific binding, are indicated on the ordinate. Days after immunization are indicated on the abscissa. After the titers of the animals significantly decreased, the rabbits were reimmunized with 50 $\mu$g of hCG-$\beta$ in an emulsion devoid of tubercle bacillus. In separate studies, animals were immunized with only 20 $\mu$g of hCG-$\beta$, resulting in a similar anamnestic responses (data not shown).

initial and "booster" immunizations of several rabbits. Antibody titers were strikingly higher after the "booster" injection. One should not assume that the antibody generated with the "booster" is identical with that generated in response to the primary immunization. One needs carefully to screen each bleed for sensitivity and specificity. In most cases, one can pool sera harvested over several weeks, but only after characterizing individual sera over that time span. As a generalization, antibody titers are readily maintained or increase with "booster" injections, but the specificity of the antisera may be lost or markedly changed with repeated injections. Obviously, there are a few exceptions to that generalization.

## Comments

The technique described herein is a modification of that initially described by Freund.[4] The intradermal technique is relatively straight-

[4] J. Freund, *Annu. Rev. Microbiol.* **1**, 291 (1947).

forward, is easy to perform, and incorporates 100 μg or less of immunogen. With this or any other immunization technique, several factors need be considered. These include selection of animal species, time to harvest antibody of highest sensitivity and specificity, selecting the appropriate time to reimmunize the animals, as well as incorporating an appropriate immunologic technique for screening the antisera for titer, specificity, and sensitivity.

# [3] Production of Specific Antisera by Immunization with Precipitin Lines

*By* JENS KRØLL



For the production of monospecific antisera it is essential that the antigens used be as pure and native as possible. One simple way to meet these requirements is to use specific antigen–antibody complexes as immunogen.[1] Passive immunodiffusion techniques can be used for this purpose.[1-7] However, these techniques are insufficient for the resolution of complex antigen–antibody systems. This requirement is better met by the more recently developed quantitative immunoelectrophoretic procedures, which in addition to a higher resolution improve the conditions for the comparison of different patterns.[8-18]

[1] S. Leskowitz, *J. Immunol.* **85**, 56 (1960).
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[18] J. Krøll, *Scand. J. Immunol.* **2**, *Suppl.* **1**, 61 (1973).







The following sections deal with the use of the line-immunoelectrophoretic procedure for the isolation of pure immunogens as well as for the evaluation of antibody titers and specificity.[14-18]

## Materials and Methods

*Line Immunoelectrophoresis.* This procedure is carried out as described elsewhere in this volume [25].

*Isolation of Precipitin Lines.* After immunoelectrophoresis the agarose gel is blotted with filter paper under a slight pressure to remove nonprecipitated antigens and to reduce the agarose gel to a thin but not completely dry sheet. The precipitin lines visualized by dark-field illumination or by staining in a dilute aqueous solution of Coomassie Brilliant Blue (0.1 g/liter) are cut out from the gel by means of a Linocutter (Fig. 1). The 8–10 cm-long narrow gel strip containing the precipitin line is transferred to a 5-ml test tube and washed three times with isotonic saline to elute remaining nonprecipitated or weakly associated antigens from the precipitate. Between washes the gel is centrifuged at 15,000 *g* for 10 min.

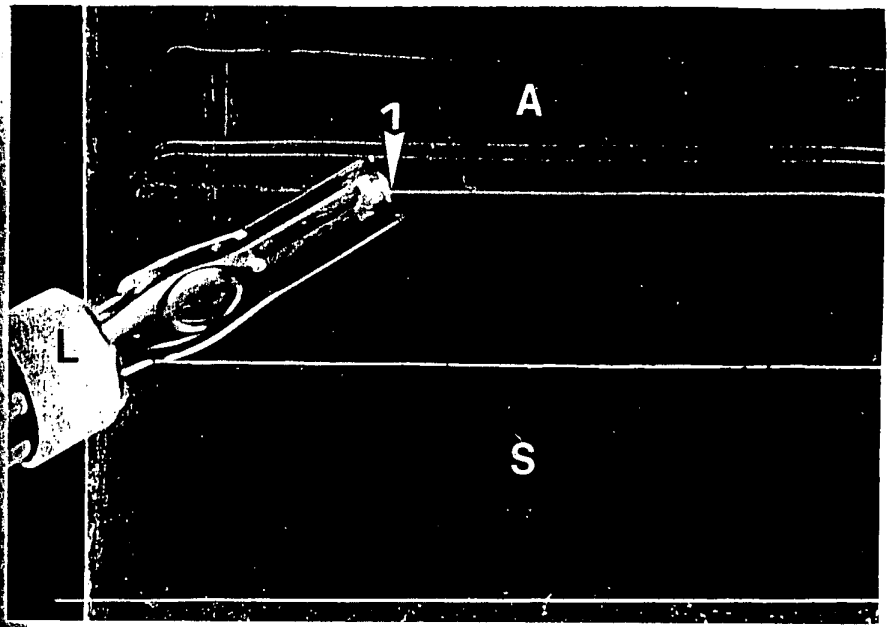


FIG. 1. Isolation of precipitin lines. S, Sample gel (1 × 20 × 70 mm) containing 0.4% of human serum; A, antiserum gel containing 3% of a polyspecific antiserum against human serum proteins. Immunoelectrophoresis was carried out at 1.5 V/cm for 20 hr. Anode is at top. The precipitin lines are visualized by dark-field illumination. One of the lines (1) is partially cut out from the gel by means of the Linocutter (L).